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CANADA

Influence of the male germ cells on egg fecundation in mullets

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Like the majority of commercial fish species with an external type of gamete union, Black Sea mullets are characterized by a high degree of fecundity and a low survival rate. The high elimination of the fish progeny in a natural environment is due to the effect of a combination of biotic and abiotic factors. However, even under optimal conditions of artificial reproduction and breeding (absence of predators, sufficient food supply, stable environmental conditions, etc.), we often observe a high mortality rate at the early stages of development (embryonic and larval). In the opinion of V.I. Vladimirov (1977), the cause of this lies in the manifestation of "critical" stages in the early development of the fish. Ichthyologists have repeatedly noted that the mass mortality of the larvae during the "critical periods" is due not only to their changing reactions to environmental factors or environmental changes, but primarily to the morphological deviations preceding them; the latter do not affect the development of the embryos and larvae at first, but they clearly do during the "critical period" (Dushkina, 1974). These irreversible changes may be caused by the inadequate environmental conditions from the time of fecundation to the onset of the critical state, as well as by even earlier changes in the germ cells due to the condition of the spawners (Ginzburg, 1953; Vladimirov, Semenov, 1959; Vladimirov, 1965; Zhukinsky, 1965, 1984).

In spawning mullet shoals, the gonads of the spawners are at the 3rd-4th and 4th stages of maturity. To obtain mature germ cells for fish breeding purposes, the fish are subjected to hormonal treatment. The heterogeneity of gonadal maturity in mullet males determines the variability of their response to pituitary injections (Mikodina, Norvillo, 1983; Kulikova, Fedulina, 1987). Because of this, it is important to study how the physiological state of the spawners influences the quality and

fecundatory capacity of the germ cells produced by them when developing the biotechnology of artificial breeding of Black Sea mullets.

This paper contains the results of experiments in the fecundation of various batches of mullet eggs with the genital products of prespawning males and fish put into a state of "functional" maturity by a pituitary injection.

Material and Method

We studied the prespawning males of the grey mullet (Am. striped mullet) and golden grey mullet (or long-finned mullet) during June—September 1980, 1982, 1986 and 1987 at the experimental station of the Azov—Black Sea Scientific Research Institute of Sea Fisheries and Oceanography (AzcherNIRO) at Zavetnoye village. The fish were caught with a grey mullet trap set up near the station in the Kerch Strait.

Some of the spawners were injected once with a pituitary suspension from the same species. The males of the grey mullet were injected with a suspension of fresh pituitaries at a rate of 18-20 mg/kg of body weight, and the golden grey mullets with a suspension of acetonized pituitaries at a rate of 3-5 mg/kg of body weight. The intact and injected fish were kept together in 2.5-3 m³ tanks (density of stocking 2-3 specimens/m³) with running aerated water with a salinity of 14-16‰ and temperature of 21-23°C for 20-24 hours. The effectiveness of these doses of hormonal preparation and the period of exposure of the males had been established in preliminary experiments. At the end of the experiment, the spawners were subjected to complete biological analysis. The age of the fish was determined by the scales. The gonad-somatic index (GSI) was calculated by the formula $\frac{Pr \times 100\%}{P_T}$ (Pravdin, 1966).

The stages of gonadal maturity were estimated visually by Sakun and Butskaya's 6-point scale (1963).

The percentage of moisture was determined in the caudal part of the testis, a test for hydration of the gonads, which characterizes the rate of spermiation (Clemens, Grant, 1964). Weighed portions of gonads (1 g) were dried to a constant weight at a temperature of 105°C.

The quality of the genital products was determined by the standard methods (Kazakov, 1978b). We determined the volume of the ejaculate (ml), the concentration of germ cells in the caudal part of the gonad (mil/mm^3), the duration of the vortical and general forward movement of the spermatozoa in the ejaculate and in caudal part of the testis.

Seventeen batches of eggs from the grey mullet and 16 from the long-finned mullet were used in the experiment. Ground testes from prespawning males without running milt and injected ripe fish with running milt served as the source of spermatozoa for artificial insemination. The caudal part of the gonad was ground up and pressed through a double gauze to obtain minced testes tissue (MTT). In some cases, the ejaculates were used for insemination, but these data are scarce, and are not presented here.

The mullet eggs were inseminated with sperm from 2-3 males separately, and in a mixture using the semi-dry method. The percentage of fecundation was determined at the stage of cleavage (2-4 blastomeres).

The quality of the sperm from intact and injected spawners of the grey mullet and long-finned mullet was compared on the basis of Student's t-test. The coefficients of correlation were calculated (Plokhinsky, 1961) to determine the degree and nature of the correlation between sperm quality and the length-weight parameters of the males, as well as the percentage of egg fecundation.

Forty males of the grey mullet and 30 of the long-finned mullet were used in the study.

Results of Investigations

Grey mullet. The spawning migration of the grey mullet lasts from June to August. Four-year-old and occasionally three- and five-year-old males form the basis of the spawning shoals. Observations over a number of years (1980-1987) to study the physiological condition of fish selected from these shoals have shown that, though they are of the same age and have similar length and weight parameters, the spawners are heterogeneous in their preparedness for reproduction. Alongside individuals with testes at the 4th stage of maturity, we encountered a

large number of fish (up to 50%) which did not exude sperm when pressure was applied to the abdomen. A study of gonads at the 4th stage of maturity has shown that the hydration of the caudal part of the gonad is weakly defined ($82.1 \pm 0.2\%$) and the density of distribution of spermatozoa in the vas deferens is high (20.3 ± 2.1 mil/mm³) in the majority of male fish. The absence of the vortical phase of movement of the spermatozoa was noted in the majority of individuals (up to 70%), and the germ cells executed only vibrational movements in 20% of the spawners studied.

Table 1. Comparative characteristics of intact and injected males of the grey mullet

Characteristic	Intact, n=19		Injected n=21		Student's criterion, td
	M±m	CV, %	M±m	CV, %	
Body length, cm	39.6±0.8	7.1	39.7±0.6	6.8	0.1
Mass, g					
body	770.0±41.0	17.9	785.0±39.0	22.0	0.3
gonads	27.9±4.0	50.5	38.0±2.8	33.0	2.1
Gonad-somatic index, %	4.0±0.5	44.0	5.3±0.2	25.0	4.1
Moisture content in					
caudal part of gonad, %	82.9±0.4	1.7	85.4±0.2	1.0	3.1
Volume of ejaculate, ml	0.3±0.1	35.0	2.8±0.8	30.0	5.6
Concentration of					
spermatozoa, mil/mm ³	19.3±1.6	56.0	15.3±0.7	46.0	2.3
Duration of spermatozoan					
movement, s					
vortical	54.0±5.0	30.6	113.0±10.0	19.0	5.4
general forward	107.0±12.0	45.0	231.0±20.0	29.0	7.5

Table 1 contains data on the intact and injected males from which testes were taken to inseminate different batches of eggs. As we can see, the spawners were similar in body length and mass. In all the other characteristics, the pituitary-injected fish injected and the "field" fish differed significantly. After pituitary treatment, we noted a considerable increase in the hydration of the gonad, as well as an increase in its mass. In the injected fish, the gonad-somatic index, the degree of hydration of the caudal part of the testis and the volume of the ejaculate were significantly higher than in the prespawning males. The concentration of spermatozoa in the caudal part of the vas deferens was lower in the injected males, which was apparently due to the thinning of the germ

cells as a result of spermiation. The movement of the spermatozoa in the vortical and consecutive phases was of much longer duration in the pituitary-injected males as compared with the "field" males. It should be said that higher variation coefficients were observed for all the characteristics (except for the moisture content in the caudal part of the gonad) with a relatively low variability of body length in all the fish studied by us. This heterogeneity of spawners of similar size was probably due to the nonuniformity of their gonadal maturity.

Table 2. Coefficients of correlation between the length-weight parameters and the degree of gonadal maturity in grey mullet males

Characteristics	Intact, n=19			Injected, n=21		
	Body length, cm	Mass, g		Body length, cm	Mass, g	
		body	gonads		body	gonads
Gonad-somatic index, %	+0.35	+0.06	<u>+0.88</u>	+0.04	+0.05	+0.71
Caudal part of gonad moisture content, %	+0.09	+0.15	+0.24	+0.30	+0.10	+0.50
concentration of spermatozoa, mil/mm ³	+0.03	+0.05	+0.19	+0.09	+0.10	+0.28
vortical movement of spermatozoa, s	+0.25	+0.11	+0.06	<u>+0.55</u>	<u>+0.46</u>	+0.34
general forward movement, s	+0.23	+0.06	+0.09	+0.24	+0.44	+0.23

Note: Underlined coefficients of correlation denote strong correlation.

During our experiments, we noted that, despite the relative heterogeneity of the males in length, the quality of their genital products depended largely on the size-weight parameters of the fish. In connection with this, we analyzed the correlation of certain indices of gonadal maturity and sperm quality with the length and mass of the body and the mass of the gonads in male fish. From the data in Table 2, we can see that only the correlation between the gonad-somatic index and the mass of the gonad is significant in prespawning males. Significant coefficients of correlation were obtained for the following characteristics in pituitary-injected specimens: between the gonad-somatic index and the degree of hydration of the caudal part of the

gonads on the one hand, and the mass of the gonad on the other; between the duration of the vortical movement of the spermatozoa and body length and mass; between the duration of the general forward movement of the spermatozoa and the mass of the fish. Apparently, the presence of positive correlations between the indices of gonadal maturity and the length-weight parameters is due to the fact that the larger males are better prepared for reproduction.

As a result of our experiments, we have established that the use of minced testes tissue from intact and pituitary-injected fish for the fecundation of eggs greatly influences the degree of egg fecundation. During individual insemination with minced testes tissue (MTT) from the caudal parts of the gonads of "field" spawners, the percentage of egg fecundation averaged 33.8%; when MTT from pituitary-injected fish was used, the percentage of fecundation averaged 74.1%. Each batch of eggs was inseminated with a mixture of genital products from 2-3 males. We found that when a mixture of sperm from intact fish was used, the percentage of fecundation averaged 50.8%, and when a sperm mixture from pituitary-injected fish was used, it averaged 76.1%. Apparently, the number of fertilized eggs depends on the quality of the spermatozoa.

In order to verify this assumption, we studied the correlation of the duration of germ cell movement, the concentration of germ cells in the caudal part of the gonad and the moisture content of the gonad with the degree of egg fecundation. We noted a highly significant correlation between the percentage of egg fecundation and the degree of hydration of the caudal part of the gonad in the pituitary-injected male fish ($r = +0.65$; $p < 0.001$). Such a correlation was not noted in the prespawning males. As indicated in Table 3, only the duration of the vortical movement of the germ cells is correlated with the percentage of fecundation in intact males. In the pituitary-injected fish, we noted a positive correlation between the duration of the vortical phase of the spermatozoa and their concentration in the caudal part of the gonad and the degree of egg fecundation. It is obvious that the quality of the germ cells and their fecundatory capacity are considerably higher in stimulated male fish as compared with "field" fish. The extent to which the physiological characteristics of the spermatozoa influence egg

fecundation in the grey mullet probably increases as they acquire the properties typical of mature germ cells in the process of "maturation".

Table 3. Coefficients of correlation between the degree of egg fecundation and sperm quality in the grey mullet

Characteristics	Intact, n=19		Injected, n=21	
	Coeff. of correlation	Level of significance	Coeff. of correlation	Level of significance
Vortical movement of spermatozoa, s	+0.48	<0.05	+0.43	<0.05
General forward movement of spermatozoa, s	+0.08	>0.10	+0.26	>0.10
Concentration of spermatozoa, mil/mm ³	+0.10	>0.10	+0.57	<0.01

Long-finned mullet. The spawning migration of this species takes place in August—September. Three- and four-year-old males form the basis of the spawning shoals. During the period of migration, the gonads in the overwhelming majority of them are at the 4th stage of maturity. At the same time, a study of a combination of morphophysiological characteristics has shown that males of the same age from the same spawning shoal and with similar body length and mass differ in their preparedness for spawning. The spermatozoa in about 20% of the fish execute only vibrational movements. In the rest, the vortical and general forward movement of the germ cells barely lasts longer than 1-2 minutes respectively. The hydration of the caudal part of the gonad is weakly defined in them ($81.4 \pm 1.0\%$), while the concentration of spermatozoa in the gonad is quite high (15.3 ± 0.8 mil/mm³).

Table 4 characterizes the spawners studied and the quality of their genital products for a combination of indices. The testes of these males were used for inseminating various batches of eggs. We can see from the data that the intact and pituitary-injected spawners were similar in a number of characteristics, namely body length and mass, the concentration of germ cells in the caudal part of the gonad, and the duration of the vortical movement of the spermatozoa. At the same time, the mass of the gonads, GSI, moisture content in the caudal part of the

gonad, volume of the ejaculate and duration of the general forward movement of the spermatozoa were significantly higher in the pituitary-injected fish as compared with the intact fish. The overwhelming majority of the fish used in the experiment were fourth-year specimens (3+). Despite the similarity in age composition, we observed a high degree of variability in such indices as the mass of the gonads, GSI, volume of the ejaculate, and the duration of germ cell movement in the vortical and general forward phases. This indicates that long-finned mullet males of the same age differ in their preparedness for reproduction.

Table 4. Comparative characteristics of intact and pituitary-injected males of the long-finned mullet

Characteristic	Intact, n=15		Injected n=15		Student's criterion, td
	M±m	CV, %	M±m	CV, %	
Body length, cm	29.0±0.4	5.3	29.8±0.5	6.4	1.3
Mass, g					
body	296.0±14.0	32.0	300.0±18.0	21.0	0.2
gonads	8.2±1.0	43.0	11.6±1.1	31.0	2.2
Gonad-somatic index, %	3.2±0.3	36.0	4.0±0.2	21.0	2.2
Content of moisture in caudal part of gonad, %	81.6±1.2	4.9	84.3±0.6	1.6	2.1
Volume of ejaculate, ml	0.2±0.1	40.0	0.8±0.2	65.0	2.7
Concentration of sper- matozoa, mil/mm ³	8.9±0.9	22.4	7.6±0.7	24.0	1.1
Duration of spermato- zoan movement, s					
vortical	53.0±6.0	35.0	65.0±7.0	30.6	1.3
general forward	166.0±20.0	50.0	329.0±28.0	40.1	4.7

Having analyzed the correlation between the length-weight parameters of the spawners and the degree of gonadal maturity, we found that only two significant coefficients of correlation exist for intact males, between the GSI and the mass of the gonad, and between the general forward movement of the spermatozoa and the mass of the gonad (Table 5). Statistically significant correlations were not noted between the other characteristics. The results obtained for the pituitary-injected males were altogether different. Here we noted that all the characteristics of gonadal maturity were clearly correlated with the

mass of the gonads. At the same time, the strength of these correlations differed. The value of the GSI and concentration of germ cells in the caudal part of the gonad showed the highest degree of dependence on the mass of the gonad. We established a direct dependence of the GSI, the content of moisture in the caudal part of the gonad and the duration of the general forward movement of the spermatozoa on the length and mass of the body in male fish. It is possible that the rather weak correlation noted in some cases between these characteristics may be due to the scarcity of study material, and will require further investigation with a larger number of specimens.

Table 5. Coefficients of correlation between the length-weight parameters and the degree of gonadal maturity in the males of the long-finned mullet

Characteristics	Intact, n=19			Injected, n=21		
	Body length, cm	Mass, g		Body length, cm	Mass, g	
		body	gonads		body	gonads
Gonad-somatic index, %	+0.14	+0.15	<u>+0.88</u>	<u>+0.55</u>	<u>+0.53</u>	<u>+0.87</u>
Caudal part of gonads moisture content, %	+0.07	+0.05	+0.36	<u>+0.59</u>	+0.20	<u>+0.42</u>
concentration of spermatozoa, mil/mm ³	+0.01	+0.10	+0.39	+0.34	+0.14	<u>+0.86</u>
vortical movement of spermatozoa, s	+0.31	+0.27	+0.35	+0.15	+0.10	+0.44
general forward movement of spermatozoa, s	+0.35	+0.29	<u>+0.46</u>	<u>+0.50</u>	<u>+0.46</u>	<u>+0.54</u>

Note. Underlined coefficients of correlation denote a significant correlation.

As a result of our experimental study of the effect of germ cell quality on egg fecundation, we have established that the percentage of fecundation averages 50% in intact fish and 65% in pituitary-injected fish when minced testes tissue from individual fish is used. In the experiment on egg fecundation with a mixture of sperm from 2-3 males, the number of fertilized eggs was somewhat higher (60 and 70% respectively). The higher percentage of fecundation in the cases where minced testes tissue from pituitary-injected males was used can

probably be attributed to the higher physiological value of their spermatozoa.

Table 6. Coefficient of correlation between the degree of egg fecundation and sperm quality characteristics in the long-finned mullet

Characteristics	Intact, n=15		Injected, n=15	
	Coeff. of correlation	Level of significance	Coeff. of correlation	Level of significance
Vortical movement of spermatozoa, s	+0.54	<0.05	+0.66	<0.01
General forward movement of spermatozoa, s	+0.11	>0.10	+0.18	>0.10
Concentration of spermatozoa, mil/mm ³	+0.49	>0.05	+0.50	<0.05

We analyzed the correlation of the qualitative and quantitative characteristics of the sperm and the degree of hydration of the caudal part of the gonad with the degree of egg fecundation, and have established that a positive relationship exists between the percentage of egg fecundation in "field" and pituitary-injected fish and the moisture content in the caudal part of the gonads ($r = +0.53$, $p < 0.001$ and $r = +0.77$, $p < 0.001$). As indicated in Table 6, egg fecundation in intact and pituitary-injected specimens correlates weakly only with the duration of the general forward movement of the spermatozoa. The correlations with the other characteristics are significant. The highest coefficient of correlation is with the duration of the vortical movement of the germ cells. Most likely, the fecundatory capacity of the spermatozoa is determined to a greater extent by the duration of the vortical phase. During this period, we observe an insignificant number of vibrating and immobile germ cells, which increases considerably as the spermatozoa pass from a vigorous vortical movement to a slowed forward one.

Discussion of Results

Earlier investigations have shown that mullet males from the same spawning shoal and with gonads at the 4th stage of maturity differ considerably in their sperm properties. Significant variability in sperm

quality is also observed in fish treated with a pituitary suspension (Mikodina, Norvillo, 1983; Kulikova, Fedulina, 1987). The same has been noted in other species of fish (Persov, 1953; Volodin, 1961; Turdakov, 1962, 1968; Zhukinsky, 1965, 1984; Ginzburg, 1968; Kazakov, 1978a; Kiselyov, 1980, etc.).

According to the data of A.F. Turdakov (1962) and A.N. Kuz'min et A.M. Chuvatova (1970), the number of physiologically full-value spermatozoa in the ejaculate depends on the maturity coefficient of the males, the concentration of milts, as well as the size, condition factor and age of the spawners. As a result of our correlation analysis, significant positive correlations have been established between the degree of gonadal maturity and the body length and mass and gonad mass in the males of the grey mullet and long-finned mullet. The difference in the quality of the spawners is probably due to a difference in the rate of gonadal maturation in fish of different size. Many authors have noted a direct correlation between growth and the rate of sexual maturation, as well as the dependence of the maturity coefficient (characteristics of the rate of maturation) on the rate of increase of body mass (Zhukinsky, 1965; Turdakov, 1972; Kiselyov, 1980, etc.). This type of dependence is apparently characteristic of mullet spawners as well.

As a result of our investigations, we have established that the duration of the general forward movement of the spermatozoa and their fecundatory capacity are much smaller in prespawning mullet males than in fish in which functional maturity is induced. It is obvious that the spermatozoa in testes at the 4th stage of maturity can be activated and are capable of fertilizing eggs. At the same time, they are not physiologically full-value cells, since they have not completed the "maturation" process which takes place under the influence of substances in the sperm that is produced at a high rate in different parts of the genital tract during the transition of the males from the potential (4th) stage of maturity to the functional (5th) stage. Apparently, pituitary injections of mullet spawners induces spermiation, during which not only sperm is secreted, but the properties of the spermatozoa themselves undergo change.

Our experimental research has shown that egg fecundation is closely related to the quality of the spermatozoa involved in fecundation. A

direct relationship has been established between the duration of the vortical phase of the spermatozoa and their fecundatory capacity. A statistically significant correlation has not been established with the duration of the general forward movement of the spermatozoa. This is probably due to the fact that the sperm contains germ cells of different age (degree of maturity), and, as they undergo hydration, the "old" spermatozoa lose their fecundatory capacity sooner than the young ones (Ginzburg, 1953, 1968; Turdakov, 1972). According to our observations, the number of vibrating and immobile spermatozoa during the first 40-60 seconds of vortical movement is insignificant. After this initial period, the number of vibrating and inactivated spermatozoa increases, particularly in low-quality sperm. A significant positive correlation is noted between the degree of egg fecundation and the number of germ cells in the caudal part of the gonad in mullets. As in other species of fish (Turdakov, 1972), the effectiveness of insemination of mullets in artificial and natural conditions depends largely on the fecundity of the males, their reproductive capacity.

As our research has shown, the degree of egg fecundation in mullets is highly dependent on the moisture content in the caudal part of the testis. This dependence is probably determined by the effect of spermiation on the degree of maturity of the spermatozoa. It is a known fact that, in the process of intensive sperm production, the hydration of the gonad increases considerably, particularly in its caudal part which is the "collector" of ripe spermatozoa (Turdakov, 1972; Belova, 1983).

We should note that the largest number of significant correlations between the characteristics of sperm quality and the percentage of egg fecundation in the males of the grey mullet has been established in those injected with a pituitary suspension. In prespawning grey mullets, we have noted only one coefficient of correlation, i.e. between the duration of the vortical movement of the spermatozoa and the degree of egg fecundation. However, in the spawners of the long-finned mullet, the same number of significant positive correlations is noted in both intact fish, and those injected with pituitary suspension. This can probably be attributed to the fact that egg fecundation in the grey mullet is greatly influenced by the degree of maturity of the spermatozoa, which is determined by the physiological condition of the males in spawning

shoals. As mentioned earlier, the gonads in grey mullet spawners are usually at the 3rd-4th stages of maturity. Their sperm is of a low quality, but it improves considerably after pituitary injections. In intact males of the long-finned mullet, the germ cells are more active and fecundative than in the grey mullet. In long-finned mullet catches, we encounter fish with running milt, which is not the case during the spawning migration of the grey mullet. Hormonal injections of long-finned mullet spawners do not increase the fecundatory capacity of their spermatozoa to the same extent as in the males of the grey mullet. The preparedness for spawning is probably much higher in the prespawning males of the long-finned mullet. It is possible that this difference is due to the species characteristics having to do with the rate of gonadal maturation, the order of migration to the spawning grounds and its duration, the sequence of joining the spawning group, as well as the formation and discharge of the ejaculate.

The results obtained in the various experiments on mullet egg insemination are of practical interest. They have shown that, in the cases where a mixture of genital products from 2-3 males was used, the percentage of fecundation was somewhat higher than in the cases where the sperm of individual spawners was used. This is probably due to the fact that, when the spermatozoa of several males are mixed, the low activity of one of them is compensated by the higher activity of the other. The use of a mixture of genital products from intact fish will increase the percentage of egg fecundation to a much greater extent than when a mixture of sperm from stimulated males is used. This is probably due to the fact that the spermatozoa in prespawning adult males are of more diverse quality, and the "compensation" in them is more manifest. After hormonal stimulation, the variability in the duration of the vortical and general forward movement of the germ cells decreases considerably, while their fecundatory capacity increases.

Summary

1. A direct positive correlation has been established between the gonadal maturity characteristics and the length-weight parameters in

the males of the grey mullet and long-finned mullet. Large mullet spawners of the same age are more prepared for reproduction.

2. Pituitary injections of grey mullet and long-finned mullet males result in a substantial increase of the GSI, the degree of hydration of the caudal part of the gonad, the volume of the ejaculate and the duration of the fecundatory phases of the spermatozoa. The quality of the germ cells in mullet males treated with a pituitary suspension is considerably higher than in intact fish.

3. The fecundation of mullet eggs is closely related to the physiological characteristics of the spermatozoa involved in fecundation. Positive coefficients of correlation have been established between the degree of egg fecundation and the duration of the vortical movement of the germ cells and their concentration in the caudal part of the testis.

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